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13. ABSTRACT ***(Maximum 200 Words)***

The Brn-3b POU family transcription factor is over-expressed in breast cancer cells compared to normal mammary cells. Moreover, the levels of Brn-3b in individual tumours show an inverse correlation with the expression levels of the BRCA-1 anti-oncoprotein. In this project, we have demonstrated that Brn-3b represses the activity of a 500 base pair fragment of the BRCA-1 gene, which contains two distinct BRCA-1 promoters (α and β). Moreover, we have shown using further constructs that the α and β promoters are independently repressed by Brn-3b.

We have also prepared human breast cancer MCF-7 cell lines, which have been stably transfected with vectors expressing the sense or anti-sense strand of Brn-3b. These cell lines exhibit respectively increased or decreased Brn-3b levels compared to cells transfected with empty expression vector. The cells with enhanced Brn-3b levels show enhanced growth rates and saturation density compared to the control cells, whilst the cells with reduced Brn-3b levels show reduced growth rate and saturation density. These results thus establish, for the first time, the ability of Brn-3b to modulate the growth of breast cancer cells and its ability to activate the BRCA-1 α and β gene promoters.

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Regulation of BRCA-1 Gene Expression and Mammary Tumourigenesis by the Brn-3b POU Family Transcription Factor

Introduction

This project is based on our finding that the Brn-3b POU family transcription factor is over-expressed in breast cancer cells compared to its expression in normal mammary epithelial cells. Moreover, we demonstrated that over-expression of Brn-3b correlated with reduced expression of the BRCA-1 anti-oncogene in the breast tumour cells (1). The aim of this project is therefore to evaluate the role of Brn-3b in regulating the general growth and gene expression pattern of breast cancer cells and its role in particular in regulating BRCA-1 gene expression.

Body

Our project has two specific tasks. Progress against each of these tasks is listed below:

(1) Task 1

Analysis of the mechanism by which Brn-3b inhibits the BRCA-1 promoter.

In our previous experiments we demonstrated that Brn-3b strongly represses the activity of the BRCA-1 promoter in co-transfection assays (1). This provides a mechanism for the inverse correlation between Brn-3b and BRCA-1 gene expression in mammary tumour samples (see above) and suggested that Brn-3b may play an important role in regulating BRCA-1 gene expression in mammary cells.

In view of the importance of the regulation of the BRCA-1 gene promoter by Brn-3b, one of our tasks was to further characterise this effect. In initial experiments, we showed that the repression of the BRCA-1 promoter by Brn-3b could be observed in co-transfection experiments with a construct, which contains only 500 bases of the BRCA-1 promoter sequence. This significantly extends our initial observation, which involves a construct containing 3,500 bases of promoter sequence. Hence, the sequences mediating repression by Brn-3b must be contained within this relatively small fragment.

However, despite its small size, this BRCA-1 gene fragment contains two distinct promoters, the α and β promoters, which can both independently drive BRCA-1 gene expression (2). We therefore tested constructs containing the isolated α or β promoters for their response to Brn-3b. In these experiments (see Appendix, Fig.1) both the construct containing the isolated α promoter and the construct containing the isolated β promoter were strongly repressed by Brn-3b. Hence, repression by Brn-3b is a property shared by these two independent promoters driving BRCA-1 gene expression.

In these experiments we also tested the effect on the α and β promoters of the related POU family transcription factor Brn-3a. The Brn-3a factor exists in two alternatively spliced forms, the longer form containing 84 additional amino acids, which are not found in the shorter form and acting as a strong transcriptional activator. In our previous work (1) we had demonstrated that the short form of Brn-3a was also able to repress the BRCA-1 promoter. This effect was noted also in our experiments with the isolated α and β promoters (see Appendix, Fig.1). Most interestingly however, in these experiments we tested for the first time the effect of the long form of Brn-3a. In these experiments (see Appendix, Fig.1) we showed a differential effect of this long form of Brn-3a on the two promoters. Although the β promoter was repressed by the long form of Brn-3a (although not as strongly as by the short form) the long form was able to activate the α promoter representing the first time that any BRCA-1 promoter has been demonstrated to be activated by a member of this transcription factor family.

Hence, the regulation of BRCA-1 gene expression by these POU family transcription factors appears to be complex with the β promoter being repressed by both forms of Brn-3a and by Brn-3b, whilst the α promoter is activated by the long form of Brn-3a and repressed by its short form and by Brn-3b. These experiments have therefore uncovered a new role for the long form of Brn-3a in activating BRCA-1 α promoter as well as further defining the elements required for repression by Brn-3b and showing that both the promoters driving BRCA-1 expression can be repressed by Brn-3b.

(2) Task 2

Characterisation of the effects of manipulating Brn-3b expression on the expression of BRCA-1 and the proliferation of normal and malignant mammary cells.

In view of the over-expression of Brn-3bin breast cancer cells compared to normal mammary epithelium, we wished to observe the effects of manipulating its expression in mammary cells on their growth rate and pattern of gene expression. To do this, we have used the human breast cancer cell line MCF-7. These cells have been transfected either with an expression vector capable of directing the over-expression of Brn-3b or with a vector containing the anti-sense strand of Brn-3b, which should reduce the endogenous Brn-3b expression. Parallel samples of cells were also transfected with empty expression vector. Following transfection, stably transfected cells were isolated and cell lines grown.

Two distinct cell lines of each type were isolated and their Brn-3b levels measured. As illustrated in Appendix Fig.2, clear over-expression of Brn-3b of approximately ten-fold was observed in the cells transfected with the Brn-3b expression vector compared to the level observed in control transfected cells. Conversely, the cells transfected with the anti-sense Brn-3b construct showed abolished Brn-3b expression. Hence, we have successfully prepared cell lines derived from the MCF-7 breast cancer cell line, which have respectively elevated or reduced Brn-3b levels compared to the levels observed in the parental cells.

Having established that the clones did exhibit the appropriate changes in Brn-3b expression, we wished to test the effect of this alteration on their growth. As indicated in Appendix Fig.3a and Fig.4, the clonal cell lines over-expressing Brn-3b showed both enhanced population doubling times as well as enhanced cellular density at the plateau phase

compared to cells transfected with empty expression vector. Conversely, the cells with reduced Brn-3b levels due to transfection with the anti-sense plasmids showed a reduced growth rate and cellular density at plateau phase compared to the control cells. These results were confirmed in saturation density growth limitation studies (see Appendix, Fig.3b and Fig.4) in which the Brn-3b over-expressing cells showed enhanced saturation density and the anti-sense cells showed reduced saturation density compared to the control cells.

These data therefore demonstrate for the first time that the Brn-3b transcription factor does indeed have a critical role in regulating the growth of mammary cells. Thus, enhancing its expression results in enhanced growth rate of the MCF-7 cells whilst the reduction in its expression conversely dramatically reduces the growth rate. Further studies on the growth characteristics of these cells, notably their rate of DNA synthesis (as measured by tritiated thymidine incorporation) and their anchorage dependence in soft agar are currently in progress.

As well as demonstrating the critical role of Brn-3b in regulating mammary cell growth, the availability of these cells will allow us to probe in great detail the mechanism by which Brn-3b regulates this growth. Thus, for example, it will be possible to perform a global analysis of gene expression in these cells compared to the control cells and identify genes whose expression is modulated by Brn-3b. This system will thus provide unique insights into the role of Brn-3b in mammary tumourigenesis and allow an elucidation of the manner in which it controls the growth of breast cancer cells.

Key Research Accomplishments

- Demonstration that both the α and β BRCA-1 gene promoters are independently regulated by Brn-3b.
- Demonstration that the long form of the related POU family transcription factor Brn-3a can activate the α but not the β BRCA-1 promoter.
- Isolation of MCF-7 human breast cancer-derived cell lines with enhanced or reduced levels of Brn-3b, compared to the parental cells.
- Demonstration that cells with enhanced Brn-3b levels show enhanced growth rates and saturation density, whereas cells with reduced Brn-3b levels show reduced growth rates and saturation density, thereby demonstrating for the first time the critical role of Brn-3b in regulating breast cancer cell growth.

Reportable Outcomes

A paper reporting the effect of Brn-3b on the growth rate of MCF-7 cells is currently in preparation.

MCF-7-derived cell lines over-expressing Brn-3 or exhibiting reduced Brn-3b levels have been isolated as envisaged in the original programme and will now be used for further experiments.

Conclusions

Our work with MCF-7 cells has shown for the first time that Brn-3b is a critical factor in regulating the growth of breast cancer cells. Simply by over-expressing Brn-3b one can enhance the growth of these cells and, perhaps more important, by reducing its expression one can conversely reduce their growth. This suggests that Brn-3b is a critical mediator controlling the growth of breast cancer cells by modulating the expression of other target genes. Future experiments will focus on identifying these target genes by global analysis of gene expression in the MCF-7 cell lines and subsequently it would be possible to determine whether the genes identified in this way, show changes in expression in breast cancer cells. These results not only therefore further characterise the mechanisms regulating gene expression and cellular growth in breast cancer cells but establish Brn-3b as a potential target for therapeutic interventions aimed at reducing its expression and/or activity in order to reduce the growth of breast tumours.

References

(1) Budhram-Mahadeo, V. S., Ndisang, D., Ward, T., Weber, B. L., and Latchman, D. S. The Brn-3b POU family transcription factor represses expression of the BRCA-1 anti-oncogene in breast cancer cells. Oncogene **18**: 6684-6691, 1999.

(2) Xu, C.F., Chambers, J.A. and Solomon, E. Complex regulation of the BRCA-1 gene. Journal of Biological Chemistry **272**: 20994-20997, 1997.

APPENDIX - FIGURES

Figure 1

Regulation of the BRCA-1 α and β promoters by Brn-3 factors in MCF-7 cells. Results of co-transfecting constructs in which the BRCA-1 α (Panel A) or BRCA-1β (Panel B) promoters drive the expression of a luciferase reporter gene. The results are shown for co-transfections involving expression vector lacking any insert (LTR) or expressing Brn-3a long form, Brn-3a short form or Brn-3b.

Figure 2

Levels of Brn-3b in cell lines transfected with the indicated plasmid and assayed by Western blotting using an antibody to Brn-3b. The column headed densitometry shows the results obtained by densitometric scanning in each case with levels equalised for that of the actin control protein, whilst the Table headed fold-expression compares the expression in each cell line to that of control cells transfected with empty expression vector (p LTR).

Figure 3

Growth rate (Panel A) and saturation density limitation (Panel B), studies of MCF-7 stable clones. Two clones over-expressing Brn-3b (Z and Y) control clones (B and C) or anti-sense clones (A1 and A2) were used in each of these experiments. The number of cells at each time point represents the mean of three independent experiments and the area bars indicate the standard deviation of the mean. * Represents statistically differences ($p \leq 0.05$) when over-expressing or anti-sense cell lines were compared to empty vector controls.

Figure 4

Summary of the results illustrated in Fig.3 showing the cell number obtained at the plateau phase in growth curve experiments and the saturation density of each cell line in saturation density limitation experiments.

FIGURE 1A

BRCA1 alpha promoter transfected with Brn-3a(l), Brn-3a(s) and Brn-3b

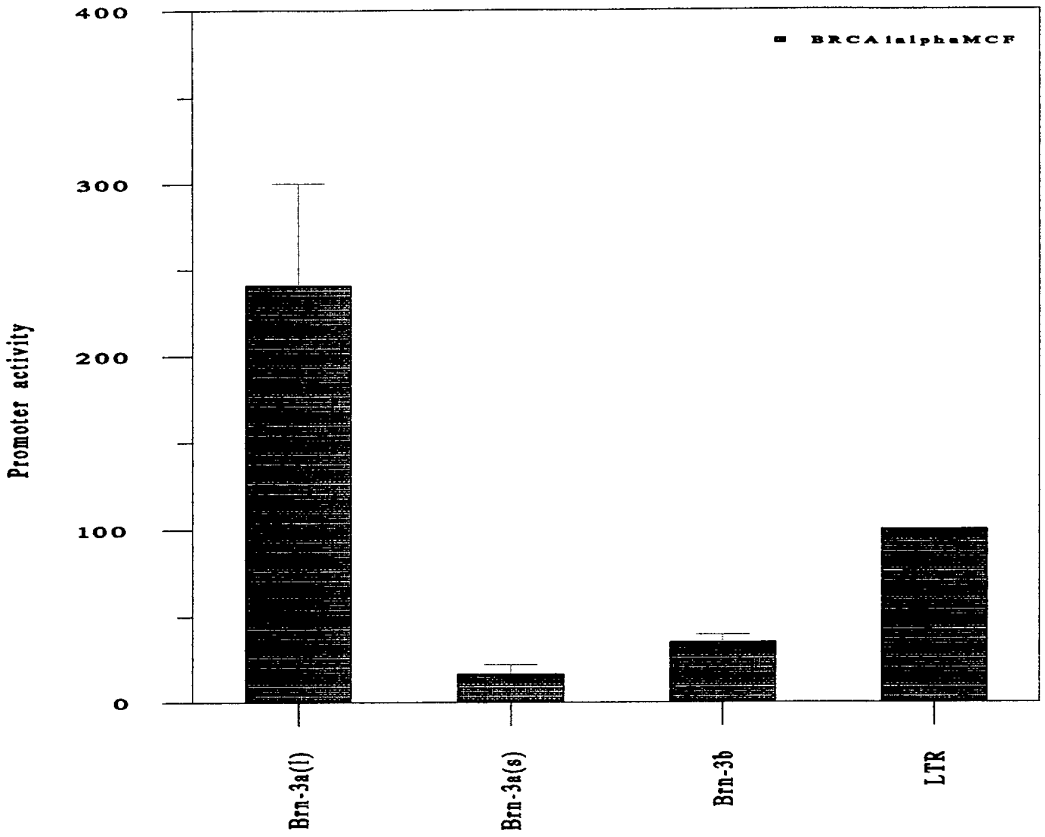

FIGURE 1B

BRCA1 beta promoter in MCF7 cells

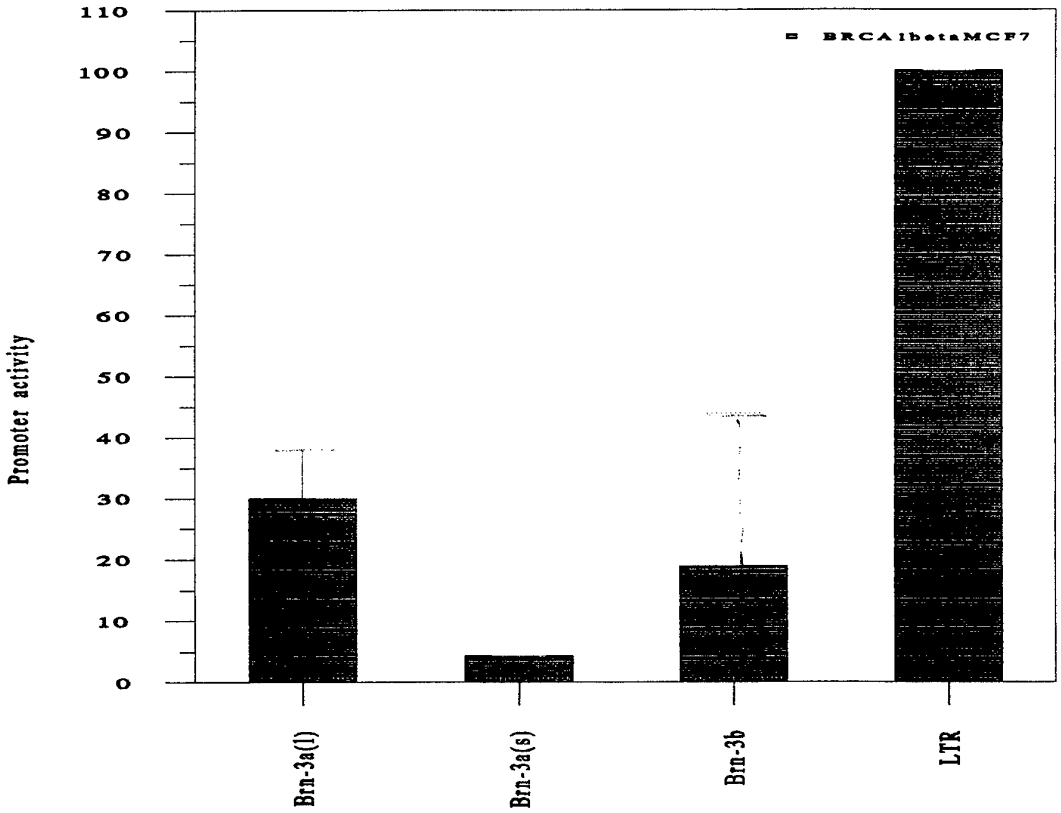

FIGURE 2

CLONE	DENSITOMETRY (arbitrary units)	FOLD EXPRESSION
	Brn-3b	Brn-3b
pLTR Brn-3b Z	41149	10.6
pLTR Brn-3b Y	45958	11.9
pLTR B	3670	1.0
pLTR C	4072	1.0
pJ4 Brn-3b antisense A1	0	0.0
pJ4 Brn-3b antisense A2	0	0.0

FIGURE 3

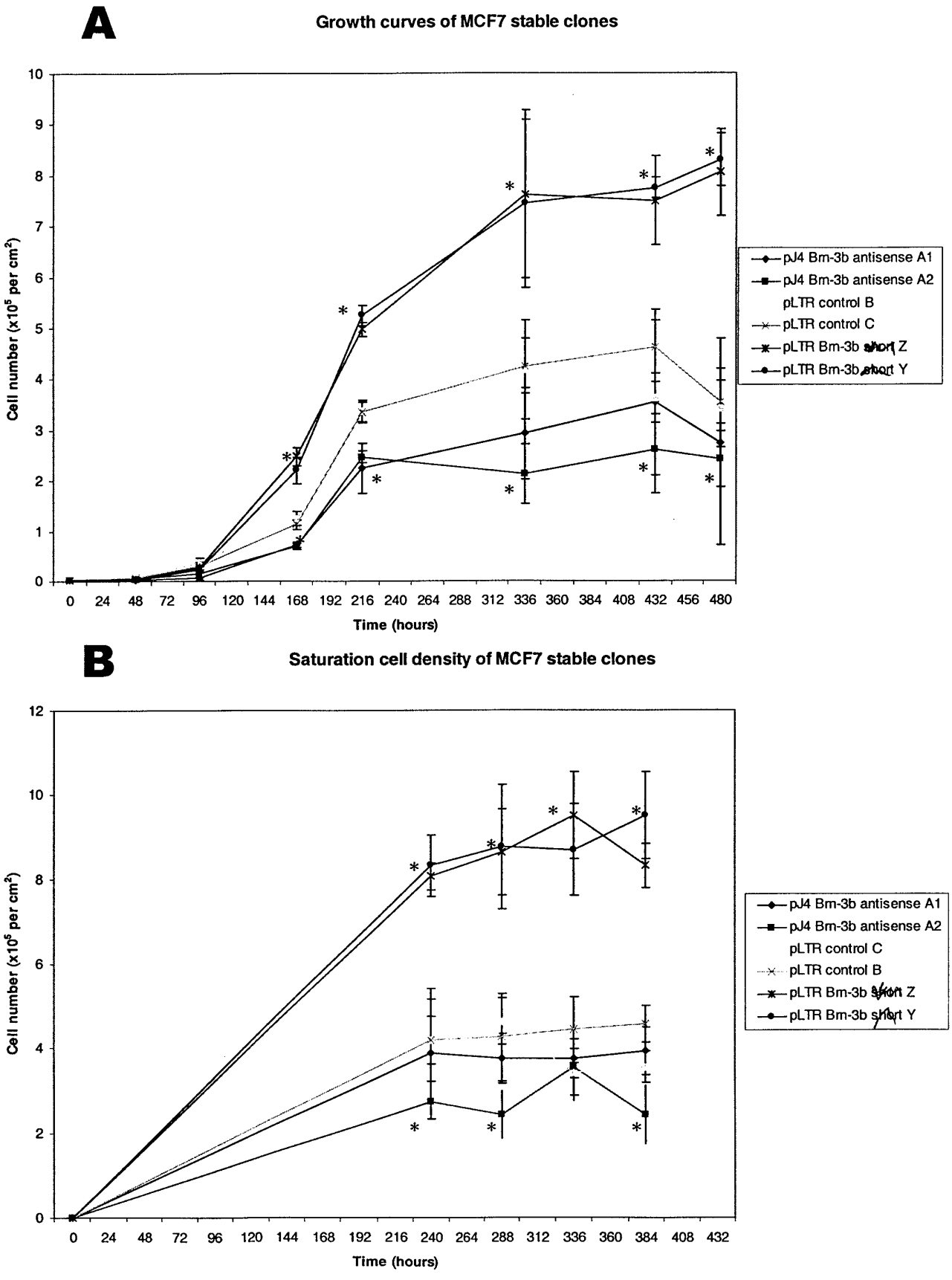
A
Growth curves of MCF7 stable clones
Cell number (x10^5 per cm^2)
Time (hours)
pJ4 Bm-3b antisense A1
pJ4 Bm-3b antisense A2
pLTR control B
pLTR control C
pLTR Bm-3b short Z
pLTR Bm-3b short Y
B
Saturation cell density of MCF7 stable clones
Cell number (x10^5 per cm^2)
Time (hours)
pJ4 Bm-3b antisense A1
pJ4 Bm-3b antisense A2
pLTR control C
pLTR control B
pLTR Bm-3b short Z
pLTR Bm-3b short Y

FIGURE 4

Growth parameters of MCF-7 stable clones

	Clone	Plateau phase		Saturation Density
		days to	cell number ($x10^5$ per cm^2)	cell number ($x10^5$ per cm^2)
Brn-3b	Z	14	7.66 +/- 0.66	8.63 +/- 0.89
Brn-3b	Y	14	7.43 +/- 0.66	8.81 +/- 1.08
Control	B	12	4.17 +/- 0.39	3.95 +/- 0.82
Control	C	12	4.25 +/- 022	4.31 +/- 0.520
Anti-sense	A1	10	2.92 +/- 0.36	3.83 +/- 0.81
Anti-sense	A2	10	2.12 +/- 0.24	2.64 +/- 0.84